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Identification and Partial Purification of Human Double Strand RNase Activity

A NOVEL TERMINATING MECHANISM FOR OLIGORIBONUCLEOTIDE ANTISENSE DRUGS*

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We have identified a double strand RNase (dsRNase) activity that can serve as a novel mechanism for chimeric antisense oligonucleotides comprised of 2'-methoxy 5' and 3' "wings" on either side of an oligoribonucleotide gap. Antisense molecules targeted to the point mutation in codon 12 of Harvey Ras (Ha-Ras) mRNA resulted in a dose-dependent reduction in Ha-Ras RNA. Reduction in Ha-Ras RNA was dependent on the oligoribonucleotide gap size with the minimum gap size being four nucleotides. An antisense oligonucleotide of the same composition, but containing four mismatches, was inactive.

When chimeric antisense oligonucleotides were pre-hybridized with 17-mer oligoribonucleotides, extracts prepared from T24 cells, cytosol, and nuclei resulted in cleavage in the oligoribonucleotide gap. Both strands were cleaved. Neither mammalian nor *Escherichia coli* RNase HI cleaved the duplex, nor did single strand nucleases. The dsRNase activity resulted in cleavage products with 5'-phosphate and 3'-hydroxyl termini.

Partial purification of dsRNase from rat liver cytosolic and nuclear fractions was effected. The cytosolic enzyme was purified approximately 165-fold. It has an approximate molecular weight of 50,000–65,000, a pH optimum of approximately 7.0, requires divalent cations, and is inactivated by approximately 300 mM NaCl. It is inactivated by heat, proteinase K, and also by a number of detergents and several organic solvents.

Antisense oligonucleotides have been shown to inhibit gene expression for a number of cellular targets (1). These compounds have proven to be effective research tools and are of interest as therapeutic agents. To date most antisense oligonucleotides studied have been oligodeoxynucleotides. Oligodeoxynucleotides are believed to cause a reduction in target RNA levels through the action of RNase H (2), an endonuclease that cleaves the RNA strand of RNA:DNA duplexes (3). This enzyme, thought to play a role in DNA replication, has been shown to be capable of cleaving the RNA component of oligodeoxynucleotide:RNA duplexes in cell-free systems as well as in *Xenopus* oocytes (4–6). RNase H is very sensitive to structural alterations in antisense oligonucleotides (7), and thus attempts to increase the potency of oligonucleotides by increasing affini-

ty, stability, lipophilicity, and other characteristics by chemical modifications of the oligonucleotide have often resulted in oligonucleotides that no longer generate substrates for RNase H when bound to their target RNA (8). RNase H activity is also somewhat variable (8), thus a given disease state may not be a candidate for antisense therapy simply because the target tissue has insufficient RNase H activity. Therefore it is clear that terminating mechanisms in addition to RNase H are of potential value to the development of antisense therapeutics.

In addition to the pharmacological inhibition of gene expression described above, it is becoming clear that organisms from bacteria to humans use endogenous antisense RNA transcripts to alter the stability of some target mRNAs and regulate gene expression (9, 10). The best characterized cases of antisense-mediated gene regulation are derived from studies on bacteria; for example an endogenous antisense RNA transcript regulates the expression of *mok* mRNA in certain bacteria. As the antisense RNA level drops, *mok* mRNA levels rise, which leads to the induction of a cytotoxic protein (*hok*), resulting in cell death (11). Other systems regulated by such mechanisms in bacteria include the RNA I-RNA II hybrid of the ColE1 plasmid (12), OOP-cII RNA regulation in bacteriophage λ (13), and the copA-copT hybrids in *Escherichia coli* (14). In *E. coli* the RNA:RNA duplexes formed have been shown to be substrates for regulated degradation by the endoribonuclease RNase III. Duplex-dependent degradation has also been observed in the archaeobacterium, *Halobacterium salinarum*, where an antisense transcript reduces expression of the early (T1) transcript of the phage gene *phiH* (15).

In bacteria, RNase III is the double strand endoribonuclease responsible for the degradation of some antisense:sense RNA duplexes. RNase III carries out site-specific cleavage of double strand RNA (dsRNA)¹-containing structures and also plays an important role in mRNA processing and in the processing of rRNA precursors into 16, 23, and 5 S ribosomal RNA (16). In eukaryotes, a yeast gene (*RNT1*) has recently been cloned that codes for a protein that has striking homology to *E. coli* RNase III and shows dsRNase activity as well as a role in ribosomal RNA processing (17). Avian cells treated with interferon produce and secrete a soluble nuclease capable of degrading dsRNA (18); however, such a secreted dsRNase activity is not a likely candidate to be involved in the intracellular degradation of antisense:sense RNA duplexes. Despite these findings, little is known about human or mammalian dsRNase activities.

In this work we have designed chimeric antisense oligonucleotides that contain 2'-methoxy-modified nucleotides in the "wings" and ribonucleotides in the "gap." These compounds bind to their cellular targets with high affinity to form an oligonucleotide:mRNA duplex in cells. Designing a series of

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¹ The abbreviations used are: ds, double strand; Ha-Ras, Harvey RAS; pCp, cytidine biophosphate.

oligonucleotides with varying ribonucleotide content enabled us to identify, and partially purify, an activity in human cells and rat liver that requires the formation of a dsRNA region (oligoribonucleotide:mRNA) to degrade target RNA in cells. The finding that human cells and rat liver contain an activity capable of recognizing and cleaving dsRNA suggests that human cells may have conserved mechanisms for regulation of gene expression by antisense RNA present in prokaryotes. Further, this activity presents a novel terminating mechanism for antisense drugs. Strategies aiming to exploit this activity to its fullest may have important implications for antisense therapeutics.

MATERIALS AND METHODS

Oligonucleotide Synthesis—RNA gap mer 2'-methoxyphosphorothioate oligonucleotides were synthesized using an Applied Biosystems 380 B automated DNA synthesizer as described previously (19). Oligonucleotides were synthesized using the automated synthesizer and 5'-dimethoxytrityl 2'-tert-butyl dimethylsilyl 3'-O-phosphoramidite for the RNA portion and 5'-dimethoxytrityl 2'-O-methyl 3'-O-phosphoramidite for 5' and 3' wings. The protecting groups on the exocyclic amines were phenoxyacetyl for riboadenosine and riboguanosine, benzoyl for ribocytosine and 2'-O-methyl A and C, and isobutyl for 2'-O-methyl G. The standard synthesis cycle was modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-methoxy. The fully protected oligonucleotide was cleaved from the support, and the phosphate group was deprotected in 3:1 ammonia/ethanol at room temperature overnight, then lyophilized to dryness. Treatment in methanolic ammonia for 24 h at room temperature was then done to deprotect all bases, and the sample was again lyophilized to dryness. The pellet was resuspended in 1 M tetrabutylammonium fluoride in tetrahydrofuran for 24 h at room temperature to deprotect the 2' positions. The reaction was then quenched with 1 M triethylaminoacetate, and the sample was then reduced to 0.5 volume by rotovac before being desalted on a G25 size exclusion column (Boehringer Mannheim). The oligonucleotide recovered was then analyzed spectrophotometrically at 260 nm for yield. Purity was characterized by capillary electrophoresis and by mass spectrometry. In all cases the purity was in excess of 90%.

³²P Labeling of Oligonucleotides—The sense oligonucleotide was 5'-end-labeled with ³²P using [γ -³²P]ATP, T4 polynucleotide kinase, and standard procedures (20). The labeled oligonucleotide was purified by electrophoresis on 12% denaturing polyacrylamide gel electrophoresis (20). The specific activity of the labeled oligonucleotide was approximately 5000 cpm/fmol.

Cell Culture and Northern Blot Analysis—T24 human bladder carcinoma cells were maintained as monolayers in McCoy's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 100 units/ml penicillin. After treatment with oligonucleotide (see below for details) for 24 h, cells were trypsinized and centrifuged, and total cellular RNA was isolated according to standard protocols (20). To quantitate the relative abundance of Ha-Ras mRNA, total RNA (10 μ g) was transferred by Northern blotting onto a Bio-Rad Zeta probe membrane (Bio-Rad) and UV cross-linked (Stratalinker, Stratagene, La Jolla, CA). Membrane-bound RNA was hybridized to a ³²P-labeled 0.9-kilobase pair Ha-Ras cDNA probe (Oncogene Science, Pasadena, CA) and exposed to XAR film (Eastman Kodak Co.). The relative amount of Ha-Ras mRNA was determined by normalizing the Ha-Ras signal to that obtained when the same membrane was stripped and hybridized with a probe for human glyceraldehyde-3-phosphate dehydrogenase (CLONTECH, Palo Alto, CA). Signals from Northern blots were quantified using a PhosphorImager and Imagequant software (Molecular Dynamics, Sunnyvale, CA).

Oligonucleotide Treatment of Cells—Cells growing as a monolayer were washed once with warm phosphate-buffered saline, then Opti-MEM (Life Technologies, Inc.) medium containing Lipofectin (Life Technologies, Inc.) at a concentration of 5 μ g/ml per 200 nm of oligonucleotide up to a maximum concentration of 15 mg/ml was added. Oligonucleotides were added and the cells were incubated at 37 °C for 4 h, after which the medium was replaced with full serum medium. After 24 h in the presence of oligonucleotide, the cells were harvested, and RNA was prepared for further analysis.

RNase H Analysis—RNase H analysis was performed using a chemically synthesized 17-base oligoribonucleotide complementary to bases +23 to +40 of activated (codon 12 mutation) Ha-Ras mRNA. 20 nm of the 5'-end-labeled RNA was incubated with a 100-fold molar excess of

the various antisense oligonucleotides in a reaction containing 20 mM Tris-Cl, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 4 units of RNase inhibitor (Pharmacia Biotech Inc.) in a final volume of 10 μ l. Secondary structures in the oligonucleotides were melted out by heating to 95 °C for 5 min, followed by slow cooling to room temperature. Duplex formation was confirmed by the shift in mobility between the single strand end-labeled sense RNA and the annealed duplex on nondenaturing polyacrylamide gels. The resulting duplexes were tested as substrates for digestion by either *E. coli* RNase HI (U. S. Biochemical Corp., Cleveland, OH) or mammalian RNase HI (partially purified from calf thymus). 1 μ l of a 1×10^{-4} mg/ml solution of either *E. coli* RNase HI or mammalian RNase HI was added to 10 μ l of the duplex reaction and incubated at 37 °C for 30 min, after which the reaction was terminated by the addition of denaturing loading buffer. Reaction products were resolved on a 12% polyacrylamide gel containing 7 M urea and exposed to XAR film (Kodak).

Cell-free in Vitro Nuclease Assays—Duplexes used in the cell-free T24 extract experiments were annealed as described above. After formation of the duplex the reaction was treated with 1 μ l of a mixture of RNase T and A (RPAII kit, Ambion, Austin, TX) and incubated for 15 min at 37 °C, to remove any nonduplexed single strand oligonucleotides. The duplex was then gel-purified from a nondenaturing 12% polyacrylamide gel. T24 cell nuclear and cytosolic fractions were isolated as described previously (21). 10 μ l of the annealed duplexes were incubated with 20 μ g of the T24 nuclear or cytosolic extract at 37 °C. The reaction was terminated by phenol/chloroform extraction and ethanol-precipitated with the addition of 10 μ g of tRNA as a carrier. Pellets were resuspended in 10 μ l of denaturing loading dye, and products were resolved on 12% denaturing acrylamide gels as described above. ³²P-Labeled 17-base RNA was base-hydrolyzed by heating to 95 °C for 10 min in the presence of 50 mM NaCO₂, pH 9.0, to generate a molecular weight ladder.

Duplexes for the rat liver extracts were prepared in 30 μ l of reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol) containing 10 nM antisense oligonucleotide and 10⁶ cpm of ³²P-labeled sense oligonucleotide. Reactions were heated at 90 °C for 5 min and incubated at 37 °C for 2 h. The oligonucleotide duplexes were incubated with either unpurified and semipurified extracts at a total protein concentration of 25 μ g of unpurified cytosolic extract, 20 μ g of unpurified nuclear extract, 1–4 μ l (1–4 μ g) ion-exchange-purified cytosolic fraction, or 1–4 μ l (100–400 ng) ion-exchange and gel filtration-purified cytosolic fractions or ion-exchange-purified nuclear fraction. Digestion reactions were incubated at 37 °C for 0–240 min. Following incubation, 10 μ l of each reaction were removed and quenched by addition of denaturing gel loading buffer (5 μ l of 8 M urea, 0.25% xylene cyanol FF, 0.25% bromophenol blue). The reactions were heated at 95 °C for 5 min and resolved in a 12% denaturing polyacrylamide gel. To perform nondenaturing gel analysis, 20 μ l of the reaction mixture were quenched by adding 2 μ l of the native gel loading buffer (50% glycerol, 0.25% bromophenol blue FF). The reactions were resolved in a 12% native polyacrylamide gel containing 44 mM Tris borate and 1 mM MgCl₂. Gels were analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Determination of 5' and 3' Termini—Nonlabeled duplex was treated with T24 extracts as described previously. Half of this reaction was then treated with calf intestinal phosphatase (Stratagene) while the other half was left untreated. The phosphatase was inactivated by heating to 95 °C, and the reactions were extracted with phenol/chloroform and then precipitated in ethanol with glycogen as a carrier. The precipitates were then treated with T4 polynucleotide kinase (Stratagene) and [γ -³²P]ATP (ICN, Irvine, CA). The samples were again extracted by phenol/chloroform and precipitated with ethanol. The products of the reaction were then resolved on a 12% acrylamide gel and visualized by exposure to Kodak XAR film. The 3' terminus of the cleaved duplex was evaluated by the reaction of duplex digestion products with T4 RNA ligase (Stratagene) and [³²P]pCp (ICN).

Liver Extraction and Preparation of Nuclear and Cytosolic Fractions—0.5 kg of rat liver was blended (Waring Commercial Blender, Dynamics Co. of America, New Hartford, CT) and homogenized (Polytron homogenizer, Brinkmann) in 5 ml of buffer X (10 mM Hepes, pH 7.5, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol/g tissue and centrifuged (Beckman centrifuge J2-21M) at 10,000 rpm for 40 min. The supernatant was precipitated with 40% ammonium sulfate (Sigma). All the activity was recovered in the 40% ammonium sulfate precipitate. The pellet was resuspended in buffer A (20 mM Hepes, pH 6.5, 5 mM EDTA, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 0.1 M KCl, 5% glycerol, 0.1% Nonidet P-40, and Triton X-100) and dialyzed to remove ammonium sulfate.

Approximately 40 g of cytosolic extract were obtained from 0.5 kg of liver.

The crude nuclear pellet was resuspended and homogenized in a glass Dounce homogenizer (Tenbroeck Tissue Grinders, Willard, OH) in buffer Y (20 mM Hepes, pH 7.5, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol). The homogenate was centrifuged at 10,000 rpm for 1.5 h. The supernatant was precipitated with 70% ammonium sulfate. The pellet was resuspended and dialyzed in buffer A. Approximately 5 g of nuclear extract were obtained.

Ion-exchange Chromatography—Nuclear and cytosolic extracts in buffer A were centrifuged at 8,000 × *g* for 10 min, and the supernatants were loaded onto Hi-Trap SP ion-exchange (Pharmacia Biotech, Sweden) columns in fast protein liquid chromatography. They were eluted with a linear gradient of NaCl, and samples were collected, directly analyzed for activity, and measured for protein concentration (Bio-Rad).

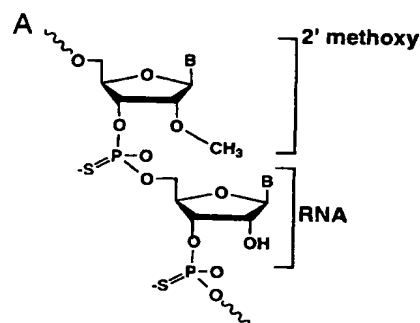
Gel Filtration High Performance Liquid Chromatography—Active samples from the ion-exchange chromatography were pooled, concentrated by a centrifugal filter device (Millipore Co., Bedford, MA), applied to a TSK G-3000 column (Toso Haas, Montgomeryville, PA) with running buffer A containing 100 mM NaCl. Samples were collected and UV absorption at 280 nm was determined; then they were directly analyzed for activity and measured for protein concentration. Concentrated fractions from the gel filtration chromatography were subjected to 12% SDS-polyacrylamide gel electrophoresis (20).

RESULTS

Chimeric 2'-Methoxy-Oligoribonucleotides (RNA GAP Mer) Mediate Digestion of Target RNA in T24 Cells—In two previous publications, structure-activity analyses of antisense oligonucleotides specific for codon 12 of the Ha-ras oncogene containing various 2'-sugar modifications were reported (22, 23). Although the 2'-modified oligonucleotides hybridized with greater affinity to RNA than did unmodified oligodeoxynucleotides, they were completely ineffective in inhibiting Ha-ras gene expression (23). The lack of activity observed with these 2'-modified oligonucleotides was attributed to their inability to create duplexes that could serve as substrates for degradation by RNase H when bound to their target RNAs (22). Because 2'-modified, and more specifically, 2'-methoxy oligonucleotides do not result in the nucleolytic degradation of their target mRNA, they provide a unique tool for the identification of novel nucleolytic activities that become activated when structural changes are introduced to fully modified 2'-methoxy antisense oligonucleotides.

In this study we have introduced ribonucleotide stretches of various lengths into the center of 17-base 2'-methoxy oligonucleotides targeting Ha-Ras mRNA, to form 2'-methoxy-ribonucleotide 2'-methoxyphosphorothioate oligonucleotides (RNA gap mers) (see Fig. 1, A and B, for structures). When hybridized to the cellular mRNA target, the resulting duplex consists of two regions that are not targets for nucleolytic degradation (the 2'-methoxy "wings") and one oligoribonucleotide:RNA duplex region that is potentially a target for a ribonuclease activity that recognizes RNA:RNA duplexes.

T24 human bladder carcinoma cells contain an activating G213T transversion mutation in the Ha-ras gene at the codon 12 position (24). Chimeric RNA gap mer antisense oligonucleotides specific for this mutation were transfected into T24 cells growing in culture. After incubation with oligonucleotides for 24 h, cells were harvested, total cytosolic RNA was isolated, and Northern blot analysis for Ha-Ras mRNA levels was performed. As previously observed, fully modified 2'-methoxy oligonucleotides did not support nucleolytic cleavage of target mRNA and therefore did not lead to a reduction in steady state levels of Ha-Ras mRNA, even at the highest concentration tested (Fig. 2A, top panel, full 2'-methoxy). An RNA gap mer oligonucleotide with only 3 ribonucleotides in the gap was also incapable of inducing nucleolytic cleavage of the target RNA (Fig. 2A, bottom panel, 3 GAP RNA). However, T24 cells treated



B H-RAS TARGETED ANTISENSE OLIGOS:

FULL 2' methoxy	□□□□□□□□□□□□□□□□□□
3 BASE RNA GAP	□□□□□□□□□□□□□□□□
5 BASE RNA GAP	□□□□□□□□□□□□□□□□
7 BASE RNA GAP	□□□□□□□□□□□□□□□□
9 BASE RNA GAP	□□□□□□□□□□□□□□□□
FULL RNA	●●●●●●●●●●●●●●●●●●

* phosphorothioate linkages throughout the oligonucleotides

FIG. 1. Structure of chimeric RNA gap mer oligonucleotides. A, chemical structures show 2 nucleosides of a chimeric 2'-methoxy-ribonucleotide oligonucleotide molecule, with a phosphorothioate linkage between the nucleotides. B, schematic shows the design and composition of oligonucleotides used in this study. Open squares represent 2'-methoxy-modified nucleotides, filled circles represent ribonucleotides. Phosphorothioate linkages are present throughout all the oligonucleotides shown.

with RNA gap mer oligonucleotides containing 5, 7, and 9 ribonucleotides in the gap as well as a full phosphorothioate oligoribonucleotide molecule displayed dose-dependent reductions in Ha-Ras steady state mRNA levels (Fig. 2B, top four panels, respectively). T24 cells treated with a control 9-base RNA gap mer oligonucleotide that contained four mismatched bases in its sequence did not show dose-dependent reduction in Ha-Ras mRNA suggesting that hybridization to the target RNA was essential for activity (Fig. 2B, bottom panel). The ability of the RNA gap mer oligonucleotides to reduce Ha-Ras mRNA was dependent on the number of ribonucleotides incorporated into the RNA gap and thus the size of the RNA:RNA duplex formed in cells. The fact that the RNA gap mer oligonucleotide containing three ribonucleotides in the gap was unable to induce reduction in target mRNA suggests that the activity involved requires an RNA:RNA duplex region of at least four ribonucleotides for cleavage of the target. T24 cells treated with 600 nM of the various RNA gap mer oligonucleotides demonstrated a reduction in Ha-Ras mRNA levels of 51 ± 8% for the 5 RNA gap mer, 49 ± 4% for the 7 RNA gap mer, 77 ± 1% for the 9-base RNA gap mer, and 38 ± 5% for the full oligoribonucleotide, respectively, when compared with nontreated controls. The full phosphorothioate oligoribonucleotide molecule was slightly less active than the RNA gap mer oligonucleotides. This is probably due to the relative decrease in stability of the full oligoribonucleotide in cells resulting from inactivation by single stranded ribonucleases, as phosphorothioate 2'-methoxy modified oligonucleotides are considerably more stable than phosphorothioate oligoribonucleotides (25). This suggests that for therapeutic purposes RNA gap mer phosphorothioate oligonucleotides protected by 2'-methoxy wings (or other even more stable 2' modifications) would be more potent molecules. These

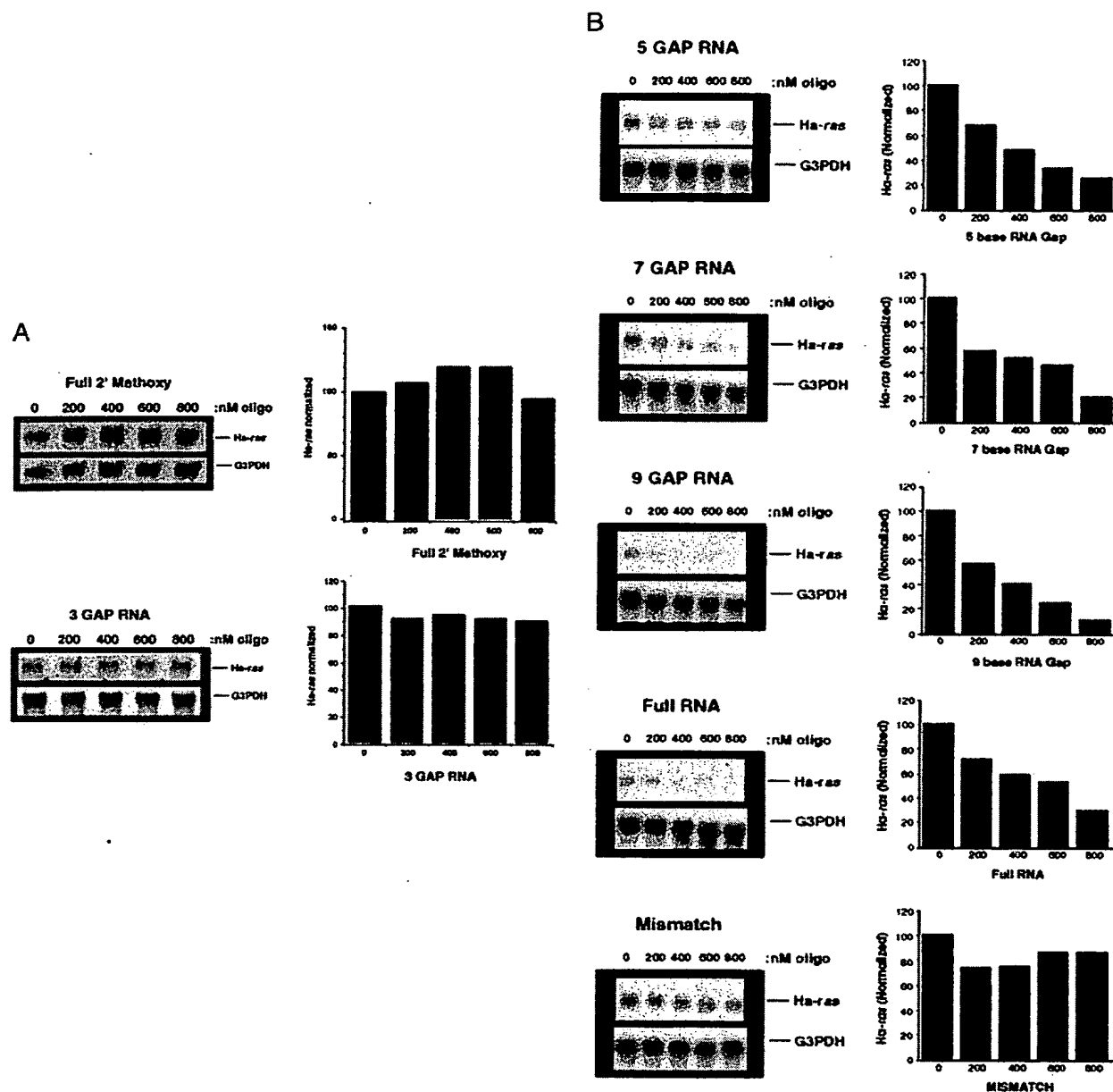


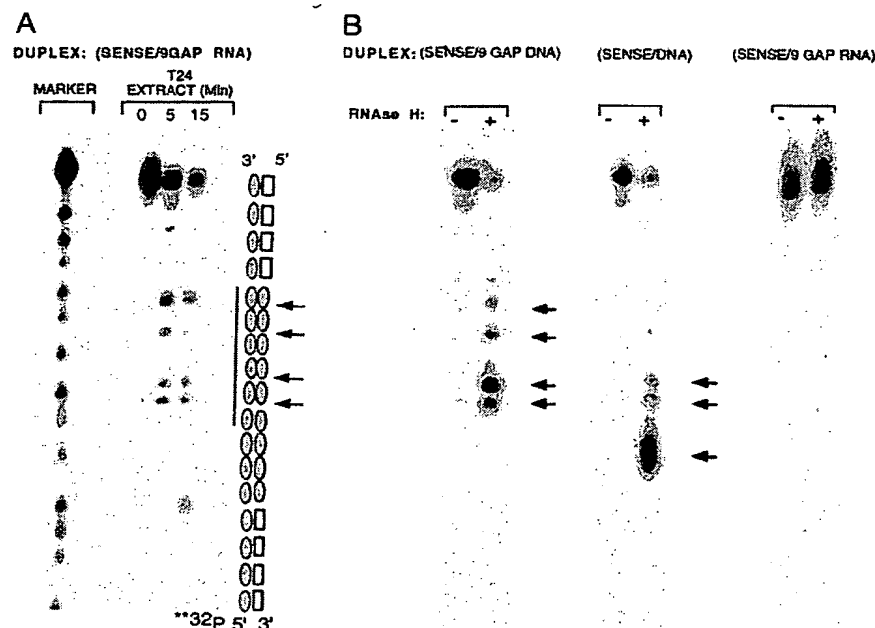
FIG. 2. Ha-Ras mRNA levels in cells treated with full 2'-methoxy or chimeric RNA gap mer oligonucleotides. A, Northern blot analyses for Ha-Ras mRNA levels in T24 cells treated with the indicated doses of full 2'-methoxy oligonucleotide (top panel) or 3-gap oligonucleotide (bottom panel) for 24 h. The upper band is the signal for Ha-Ras. This signal was normalized to that obtained for glyceraldehyde-2-phosphate dehydrogenase (G3PDH) (lower band), and relative Ha-Ras levels were determined and are presented graphically (right panel). Neither oligonucleotide treatment reduced Ha-Ras mRNA levels. B, Northern blot analyses of T24 cell treated as in A, except with chimeric RNA gap mer oligonucleotides containing either a 5, 7, or 9 ribonucleotide gap or a full ribonucleotide molecule (top four panels, respectively). Cells were also treated with a control oligonucleotide that contains nine ribose nucleosides with four mismatched bases to the Ha-Ras mRNA sequence (bottom panel). Ha-Ras signals were normalized to that of G3PDH, and relative Ha-Ras levels are shown (right panel).

experiments demonstrate that an endoribonuclease activity in T24 human bladder carcinoma cells recognizes the internal oligoribonucleotide:RNA portion of a chimeric duplex and reduces target mRNA levels.

An Activity Present in Human Cellular Extracts Induces Cleavage of RNA Gap Mer Oligonucleotide:RNA Duplex within the Internal RNA:RNA Portion in Vitro—To further characterize the dsRNA cleavage activity in T24 cells, we prepared T24 cellular extracts and tested these for the ability to cleave a 17-base pair duplex consisting of the 9-base RNA gap mer

oligonucleotide annealed to its complementary ^{32}P -end-labeled oligoribonucleotide. The ^{32}P -labeled duplex was incubated with 20 μg of cytosolic extract at 37 $^{\circ}\text{C}$ for the indicated times (Fig. 3A), followed by phenol chloroform extraction, ethanol precipitation, and separation of the products on a denaturing gel. This duplex was a substrate for digestion by an activity present in T24 extracts as can be seen by the loss of full-length end-labeled RNA and the appearance of lower molecular weight digestion products (indicated by arrows, Fig. 3A). In addition, the activity responsible for the cleavage of the duplex

FIG. 3. Effect of T24 cytosolic extracts and RNase H on duplexes *in vitro*. A, a 17-base pair duplex consisting of the Ha-Ras targeted 9-base RNA gap mer oligonucleotide annealed to a 32 P-labeled RNA complement was incubated with 20 μ g of a T24 cytosolic protein fraction for the indicated times at 37 °C, the reaction was stopped, and products were resolved on a denaturing polyacrylamide gel. Digestion products (arrows) indicate that cleavage of the duplex is restricted to the RNA:RNA region (see schematic of duplex, far right). B, the same 9-base RNA gap mer oligonucleotide:RNA duplex as in A was incubated with or without *E. coli* RNase H (–, +). The lack of digestion products indicates that this duplex is not a substrate for RNase H (right panel). Duplexes consisting of 32 P-labeled RNA annealed to either a full oligodeoxynucleotide (middle panel) or 9-base DNA gap mer oligonucleotide (left panel) are substrates for cleavage by RNase H and thus generate digestion products as expected (arrows).



displayed specificity for the RNA:RNA portion of the duplex molecule, as indicated by the sizes of the cleavage products it produced (see the physical map of the 32 P-end-labeled 9-base RNA gap mer:RNA duplex, Fig. 3A, far right). To evaluate the cellular distribution of this dsRNase activity, nuclear extracts were prepared from T24 cells and tested for the ability to digest the 9-base RNA gap mer oligonucleotide:RNA duplex. Nuclear extracts prepared from T24 cells were able to degrade the target duplex, and the activity was present in the nuclear fraction at comparable levels to that in the cytoplasmic fractions (data not shown). Cellular extracts prepared from human umbilical vein epithelial cells, human lung carcinoma (A549), and HeLa cell lines all contained an activity able to induce cleavage of the 9-base RNA gap mer:RNA target duplex *in vitro*. This activity was abolished by pretreatment of the extracts with proteinase K for 15 min at 65 °C (data not shown).

The initial RNA gap mer antisense oligonucleotides were synthesized to contain phosphorothioate linkages throughout the entire length of the molecule. As this results in increased stability to single strand nucleases, we reasoned that it would inhibit cleavage of the antisense strand by the dsRNase as well. Therefore, to determine if the activity we have described can cleave both strands in a RNA duplex molecule, we synthesized a 9-base RNA gap mer antisense oligonucleotide that contained phosphorothioate linkages in the wings between the 2'-methoxy nucleotides but had phosphodiester linkages between the nine ribonucleotides in the gap. A duplex comprised of the 32 P-labeled 9-base RNA gap mer phosphodiester/phosphorothioate antisense oligonucleotide described above and its complementary oligoribonucleotide was tested as a substrate for the dsRNase activity in T24 extracts. The activity was capable of cleaving the antisense strand of this duplex as well as the sense strand and the pattern of the digestion products indicated that cleavage was again restricted to the RNA:RNA phosphodiester portion of the duplex (data not shown).

An RNA Gap Mer Oligonucleotide:RNA Duplex Is Not a Substrate for RNase HI—To exclude the possibility that the cleavage seen might be due to an RNase H type activity, we tested the ability of *E. coli* RNase H to cleave a 17-base pair duplex composed of the 9-base RNA gap mer oligonucleotide

and its complementary 5'- 32 P-labeled oligoribonucleotide *in vitro*. As can be seen in Fig. 3B (far right panel), the 9-base RNA gap mer oligonucleotide:RNA duplex was not a substrate for RNase H cleavage as no lower molecular weight bands appeared when it was treated with RNase H. However, as expected both a full oligodeoxynucleotide:RNA duplex and a 9-base DNA gap mer oligonucleotide:RNA duplex were substrates for RNase HI under the same conditions, as is evident by the appearance of lower molecular species in the enzyme-treated lanes (Fig. 3B, left and middle panels). It is interesting to note that RNase HI cleavage of the 9-base DNA gap mer oligonucleotide:RNA duplex (Fig. 3B, left panel) and cleavage of the 9-base RNA gap mer oligonucleotide:RNA duplex by T24 cellular extracts resulted in similar digestion products (Fig. 3A). Both RNase HI and the activity in T24 cells displayed the same preferred cleavage sites on their respective duplexes. Cleavage was restricted to the 3' end of the target RNA in the region opposite either the DNA or RNA gap of the respective antisense molecule. This suggests that RNase H and the dsRNase activity described here may share binding as well as mechanistic properties.

dsRNase Activity Generates 5'-Phosphate and 3'-Hydroxyl Termini—To determine the nature of the 5' termini resulting from cleavage of the duplex *in vitro*, nonlabeled duplex was incubated with T24 cellular extracts as described previously, then reacted with T4 polynucleotide kinase and [γ - 32 P]ATP with or without prior treatment with calf intestinal phosphatase. Phosphatase treatment of the duplex products was essential for the incorporation of the 32 P label during the reaction with polynucleotide kinase, indicating the presence of a phosphate group at 5' termini of digestion products (data not shown). The 3' termini of the cleaved duplex products were evaluated by the reaction of duplex digestion products with T4 RNA ligase and [32 P]pCp. T4 RNA ligase requires a free 3'-hydroxyl terminus for the ligation of [32 P]pCp. The ability of the duplex digestion products to incorporate [32 P]pCp by T4 RNA ligase indicated the presence of 3'-hydroxyl groups (data not shown).

dsRNase Activity in Rat Liver—To determine if non-human mammalian cells contain dsRNase activity, and to provide a

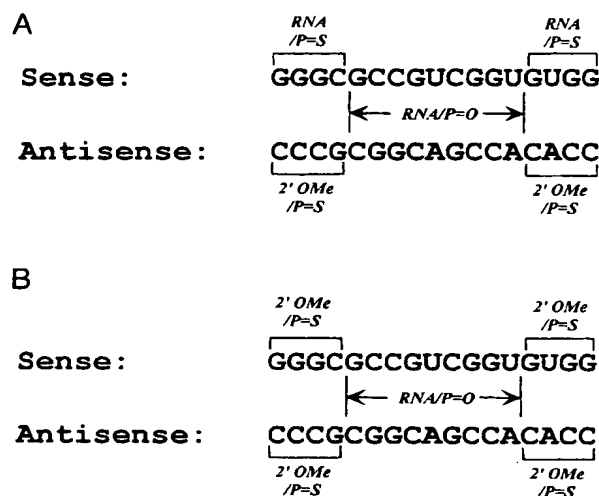


FIG. 4. Two sets of duplex oligoribonucleotide substrates for the dsRNase activity assay in nondenaturing and denaturing acrylamide gel assays. P=O, phosphodiester linkage; P=S, phosphorothioate linkage; 2' Ome, 2'-methoxy nucleoside. A, sense strand has P=S in the wings. B, sense strand was 2' Ome and P=S in the wings.

source from which the activity might be purified, we chose rat liver. In preliminary experiments, dsRNase activity was observed in rat liver homogenates, but the homogenates also displayed higher levels of single strand RNases that confounded analysis because of cleavage of the oligoribonucleotide overhangs after cleavage by dsRNase. To solve this problem, we used two additional substrates and a nondenaturing gel assay. The "antisense" strand in both substrates contained 2'-methoxyphosphorothioate wings on either side of an nine-base ribonucleotide phosphodiester gap. The "sense" strand was either an oligoribonucleotide, with phosphodiester in the 9-base gap flanked by phosphorothioate linkages (Fig. 4A), or had flanks comprised of 2'-methoxy nucleosides with phosphorothioate linkages (Fig. 4B). Both substrates were more stable to exonuclease digestion than an oligoribonucleotide, and the substrate with phosphorothioate linkages and 2'-methoxy nucleosides in both strands was extremely stable. This was important because of the abundance of single strand RNases relative to the dsRNase activity in the liver and supported the use of nondenaturing assays, as the products of the cleavage by dsRNase remained double-stranded.

Rat liver cytosolic and nuclear extracts induced cleavage of the duplex substrate (Fig. 5, lanes 2 and 3). Both extracts resulted in more rapidly migrating bands on native gel electrophoretic analyses. A dsRNase, RNase V1 cleaved the substrate (lanes 16 and 17); T24 extracts also cleaved the substrate (lanes 18 and 19). Neither bacterial nor human RNase H, nor single-strand RNases cleaved the substrate (lanes 4–15).

Fig. 6A shows the elution profile of the rat liver cytosolic extract after ion-exchange chromatography. Fig. 6B shows that the dsRNase activity eluted in fractions 53–63 (300–450 mM NaCl). In contrast, the dsRNase activity in the nuclear extract eluted at 700–800 mM NaCl (Fig. 6, C and D). In some chromatographic separations, activities that eluted at both high and low NaCl concentrations were observed in the cytosol and the nucleus.

Fractions from the ion-exchange chromatography of rat liver cytosol were concentrated and subjected to size exclusion chromatography as described under "Materials and Methods." Fig. 7A shows the elution profile and Fig. 7B the activity profile of cytosolic dsRNase after size-exclusion chromatography. Fig. 7C

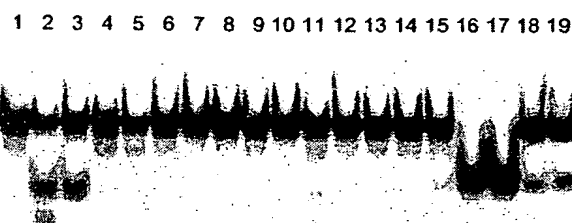


FIG. 5. Cleavage of substrates by rat liver cytosolic and nuclear extracts. Antisense and sense oligonucleotides were annealed and incubated with the cellular extracts and variety of RNases, then subjected to native 12% acrylamide gel, as described under "Methods and Materials." Lane 1, RNA duplex substrate; lanes 2 and 3, duplex digested with partially purified rat liver cytosolic (1 μ g) or nuclear extract (0.1 μ g); lane 4, RNase A (10^{-4} units); lanes 5 and 6, RNase CL3 (1 and 10^{-1} unit); lanes 7 and 8, partially purified calf thymus RNase H (1/5 and 1/50 unit); lanes 9 and 10, *E. coli* RNase H₁ (1/400 and 1/4000 unit); lanes 11 and 12, RNase T1 (10^{-1} and 10^{-2} unit); lanes 13 and 14, RNase T2 (1 and 10^{-1} unit); lane 15, RNase S1 (1 unit); lanes 16 and 17, RNase V₁ (1 and 10^{-1} unit); lanes 18 and 19, T24 cellular extract (20 and 40 μ g).

shows a polyacrylamide gel electrophoretic analysis of the concentrated active fractions, after the ion-exchange chromatography, and the fractions from the size exclusion chromatography. The fraction with greatest dsRNase activity (fraction 3) had a mean molecular mass of 45–80 kDa, and two bands at approximately 50 kDa appeared to be enhanced on polyacrylamide gel analysis. Comparison of the gel analysis of fractions 3 and 4 shows that proteins of approximately 40 and 64 kDa did not co-purify with the dsRNase activity. Lane 5 shows that a protein of approximately 55 kDa did not co-purify with the activity. Obviously, fraction 3 represents only a partially purified fraction. Table I provides a summary of the purification and recovery of dsRNase activities from nuclear and cytosolic liver extracts. Purification of the protein(s) responsible for the nuclear activity has proven more difficult and will be the subject of an additional communication.

The effects of various conditions on the dsRNase activity were evaluated using the active fractions after ion-exchange chromatography. Fig. 8 shows that dsRNase activity was apparent in a Tris or phosphate buffer at pH 7–10 (lanes 1–15). It was unstable in acetonitrile or methanol (lanes 42 and 43) and was inhibited by NaCl; dsRNase activity was inhibited by 30% at 10 mM, >60% at 100 mM, and 100% at 300 mM NaCl (lanes 36–40). Heating for 5 min at 60 °C inactivated the enzyme (lanes 21–23), and the activity had a temperature optimum of 37–42 °C (lanes 27–29). At 25 °C, the activity was approximately 50% of that observed at 37 °C (lane 30). The activity was inhibited by EDTA (lanes 31–35), required Mg^{2+} and was stable to multiple freeze/thaws (lanes 24–26). It also was ablated by treatment with proteinase K (data not shown).

Cleavage Characteristics—To characterize the site of cleavage in more detail, it was necessary to minimize single strand cleavage that occurred after endonuclease cleavage and during handling, particularly after denaturing of the duplex. Consequently, we used the most stable duplex substrate in which both strands of the duplex contained flanking regions comprised of 2'-methoxy nucleosides and phosphorothioate linkages.

Fig. 9A displays the results from native gel analyses. Lane 1 shows the position at which the ^{32}P -labeled sense strand migrated in the native gel. Lane 2 shows that the "sense" single strand was not digested by dsRNA-specific ribonuclease V1. Lanes 3 and 4 show the degradation of RNA duplexed with antisense RNA gap mer resulting from high and low concentrations of V1 RNase. Lanes 5 and 6 show that crude nuclear extract degraded the duplex in a Mg^{2+} -dependent fashion.

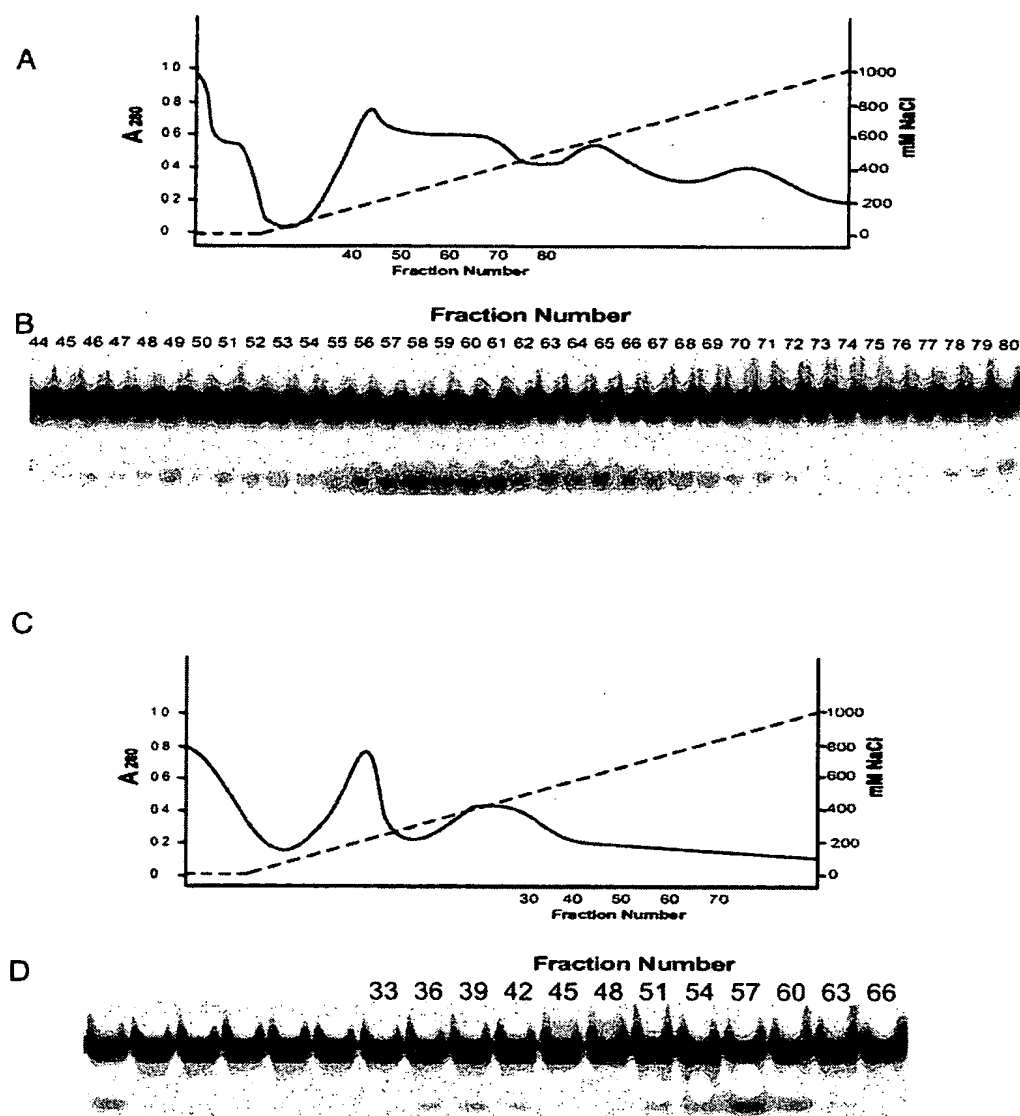


FIG. 6. Ion-exchange chromatograph of dsRNase activity from rat liver cytosolic (A and B) and nuclear (C and D) extracts. After NH_4Cl precipitation and dialysis with buffer A, the extracts were loaded onto a 100-ml Hi-Trap SP ion-exchange column and eluted by a 0–1 M NaCl increase gradient (-----). A and C, elution profile; B and D, dsRNase activity of the fraction (1–2 μl) was determined as described under "Materials and Methods."

Lane 7 shows that crude cytosolic extract also induced cleavage of the substrate. Ion-exchange purified cytosolic extract cleaved the substrate in a Mg^{2+} -dependent fashion as well (lanes 8 and 9). Active fractions alter size exclusion chromatography also cleaved the substrate in a Mg^{2+} -dependent fashion (lanes 10 and 11).

Fig. 9B shows the denaturing gel analysis of the degradation products. Lane 1 shows the products of a limit digest of the single-strand sense oligonucleotide. The position of the degradate is consistent with it being the 2'-methoxyphosphorothioate-flanking region (wing). RNase V1 digestion of the single-strand substrate resulted in little degradation (lane 2). RNase V1 digestion of the duplex resulted in degradates reflecting cleavage at several sites within the dsRNA gap (lane 3 and 4). In lanes 4–14, the band at the top of the gel demonstrates that, even after denaturation, some of the duplex remained an-

nealed, reflecting the very high affinity of duplexes comprised of 2'-methoxy nucleosides. Lanes 6–9 show that both the nuclear and cytosolic enzymes cleaved the duplex substrate at several sites within the oligoribonucleotide gap and that the sites of degradation were different from those of V1 nuclease.

DISCUSSION

By the rational design of chemically modified antisense oligonucleotides that contain oligoribonucleotide stretches of varying length, we have identified an activity in cells and rat liver that requires the formation of a dsRNA region to degrade target RNA. This activity is present at comparable levels in both the nuclear and cytoplasmic fractions of T24 human bladder carcinoma cells. We have found that this activity produces 5'-phosphate and 3'-hydroxyl termini after cleavage of its RNA substrate. The generation of 5'-phosphate and 3'-hydroxyl ter-

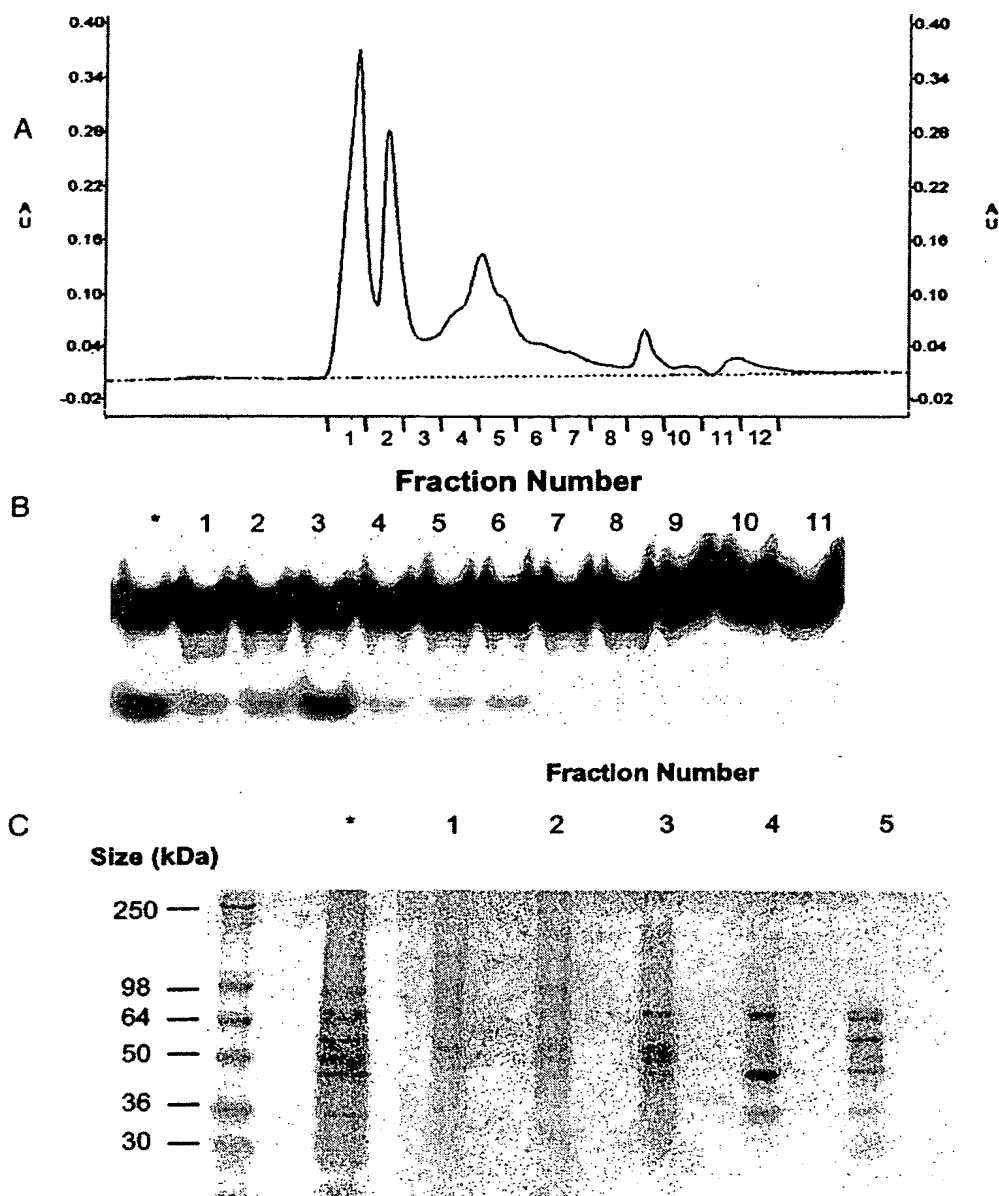


FIG. 7. Gel filtration of dsRNase activity from rat liver cytosolic extracts. Extract after ion-exchange was concentrated and loaded onto a TSK3000 gel filtration column. A, elution profile; B, dsRNase activity for the fractions (1 μ l); and C, SDS-polyacrylamide gel electrophoresis with Coomassie Blue stain (6 μ g of protein from each fraction). * = sample after ion-exchange chromatography only.

TABLE I
Partial purification of dsRNase from rat liver extracts

Fractions from rat liver nuclei and cytosol were prepared and tested as described under "Materials and Methods."

Fraction	Protein	Total activity	Specific activity	Purification factor	Recovery
	mg	unit ^a	unit/mg		%
Cytosolic extract	30,000	1,020,000	34	1	100
Ion-exchange pool	991	459,000	463	14	56
Gel filtration pool	18.4	100,980	5,600	165	22
Nuclear extract	5,000	205,000	41	1	100
Ion-exchange pool	11.2	77,900	6970	170	38

^a Unit is the amount of enzyme required to digest 10 fmol of dsRNA duplex in 15 min at 37 °C in the condition described under "Materials and Methods."

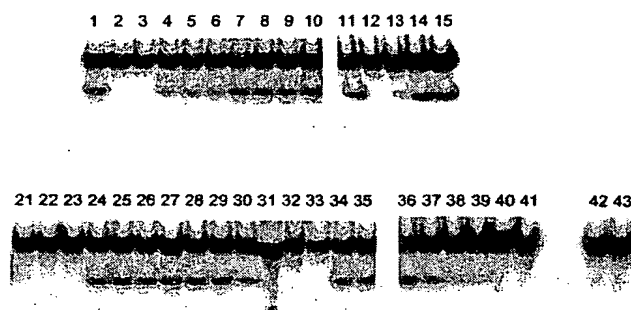


FIG. 8. Effect of various conditions on dsRNase activity. 1 μ g partially purified rat liver cytosolic extract was incubated with duplex substrate as described under "Materials and Methods." Lanes 1 and 11, 20 mM Tris buffer (pH 7.5); lanes 2–6, 20 mM sodium acetate buffer (pH 4.5, 5.5, 6.0, 7.0, and 8.0); lanes 7–10, 20 mM Tris buffer (pH 7.0, 8.0, 9.0, and 10.0); lanes 12–15, 20 mM sodium phosphate buffer (pH 5.0, 6.0, 7.0, and 8.0); lanes 16–23, 60, 80, and 100 $^{\circ}$ C, incubation of extract for 5 min prior to digestion of duplex substrate; lanes 24–26, repeat cycles of freezing and thawing 10, 3, and 0 times; lanes 27–30, digestion reaction incubated at 50, 42, 37 and 22 $^{\circ}$ C; lanes 31–35, reaction buffer with final EDTA concentration of 50, 20, 10, 5 and 0 mM; lanes 36–40, reaction buffer with final NaCl concentration of 30, 100, 300, 500, and 1000 mM; lane 41, substrate only; lanes 42 and 43, extract pretreated with organic solvent (60% methanol and acetonitrile).

mini is a common feature of several other nucleases that recognize double strand nucleic acid molecules, including RNase HI (26), the enzyme that cleaves the RNA component of a DNA:RNA duplex, and *E. coli* RNase III, which catalyzes the hydrolysis of high molecular weight dsRNA and mediates degradation of sense-antisense duplexes (27). The fact that both the oligoribonucleotide portion of the 9-base RNA gap mer strand in the 9-base RNA gap mer oligonucleotide:RNA duplex as well as the RNA strand were cleaved by this activity demonstrates that the enzyme(s) can specifically recognize and cleave both strands of an RNA:RNA type duplex. The presence of phosphorothioate linkages in the antisense molecule should prevent cleavage of this strand when administered to cells and therefore enhance the potential of such compounds to have therapeutic utility. Interestingly, cleavage of both strands does not seem to be required, in that target mRNA was greatly reduced even though phosphorothioate RNA gap mer antisense oligoribonucleotides were used.

The partial purification of the activity from liver nuclear and cytosolic extracts suggests that the activity is present in both subcellular compartments in rat liver cells as well as human cell lines. The nuclear enzyme eluted from the ion-exchange column at higher NaCl concentrations than did the cytosolic enzymes. However, both require Mg^{2+} and cleave at several sites within the oligoribonucleotide gap. Both require a duplex substrate. This may suggest that there are different types of proteins with dsRNase activity in nuclei and cytosol, but much more work is required before conclusions can be drawn. Additionally, as the nuclear activity eluted at a different NaCl concentration than did the cytosolic, it seems likely that the nuclear activity did not contribute to the cytosolic activity that eluted at lower NaCl concentrations. However, in several preparations, there was evidence of small amounts of activity that eluted at 700–800 mM NaCl in the cytosol, and this could have been due to nuclear contamination. Again, only additional work will definitively determine the cellular localization of the activities.

Many components of mRNA degradation systems have been conserved between pro- and eukaryotes (28, 29). Here we show that like some prokaryotic organisms, in which RNase III carries out the degradation of sense-antisense hybrids to regulate

the expression of some genes, human cells have conserved an activity capable of performing a similar role. For some time the dsRNA adenosine deaminase enzyme was suggested to target RNA hybrids for degradation by some unknown mechanism (30). However, more recently it has been demonstrated that deaminated transcripts are usually at least as stable as unmodified RNA (31). This enzyme efficiently modifies duplexes containing 100 base pairs or more and would therefore not be a factor in our system where dsRNA regions ranged from 3 to a maximum of 17 base pairs. In addition, Ha-Ras mRNA does not contain any adenosine residues in the region targeted by our antisense oligonucleotides. The identification of a human dsRNase activity may help us understand how human cells use endogenously expressed antisense transcripts to modulate gene expression. It also has important implications for antisense therapeutics.

The activities reported in this study appear to be novel. The properties of the proteins responsible for cleavage of the substrates are clearly different from other enzymes reported. For example, the dsRNase induced by interferon has a different molecular weight, salt and divalent ion requirements, and is secreted (18). We have not observed dsRNase H activity in cell supernatants.

The vast majority of antisense oligonucleotides used experimentally or currently being tested in the clinic are modified oligodeoxynucleotides (1, 7). It has been demonstrated that the heteroduplex formed between such oligodeoxynucleotide antisense compounds and their target RNA is recognized by the intracellular nuclease RNase H that cleaves only the RNA strand of this duplex. Although RNase H-mediated degradation of target RNA has proven a useful mechanism, it has limitations. One is the fact that the oligonucleotide must be "DNA-like," and such oligonucleotides have inherently a lower affinity for their target RNA. Strategies designed to circumvent this lower affinity include the design of gap mer oligonucleotides that are comprised of a stretch of high affinity chemically modified oligonucleotides on the 5' and 3' ends (the wings) with a stretch of deoxynucleotides in the center (the gap) (7, 23). DNA gap mer oligonucleotides have significantly higher affinities for their target. However, depending on the size of the DNA gap, RNase H activity may also be compromised (7, 23). The cellular localization and tissue distribution of RNase H activity are also concerns for antisense therapy. RNase H activity is primarily localized to the nucleus (32), although it has been detected at lower levels in the cytoplasm. RNase H activity is also variable from cell line to cell line and between tissues (8), thus a given disease state may not be a good candidate for antisense therapy, simply because the target tissue has insufficient RNase H activity. Finally, and perhaps most importantly, the majority of sites within RNA targets that have been studied are not sensitive to RNase H-induced cleavage (8). It is clear then that alternative terminating mechanisms to RNase H activation are required for widespread application of antisense therapeutics.

The activity described in this work is attractive as an alternative terminating mechanism to RNase H for antisense therapeutics. The activity relies upon "RNA-like" oligonucleotides that have higher affinity for their target and thus should have higher potency than "DNA-like" oligonucleotides. The presence of the activity in both the cytoplasm and the nucleus suggests that it might be used to inhibit many RNA processing events from nuclear pre-mRNA splicing and transport to the degradation of mature transcripts in the cytoplasm. As we have examined the dsRNase activity induced only by the RNA gap mer oligonucleotides targeted to codon 12 of Ha-Ras, it is difficult to estimate the relative abundance of this dsRNase activity or

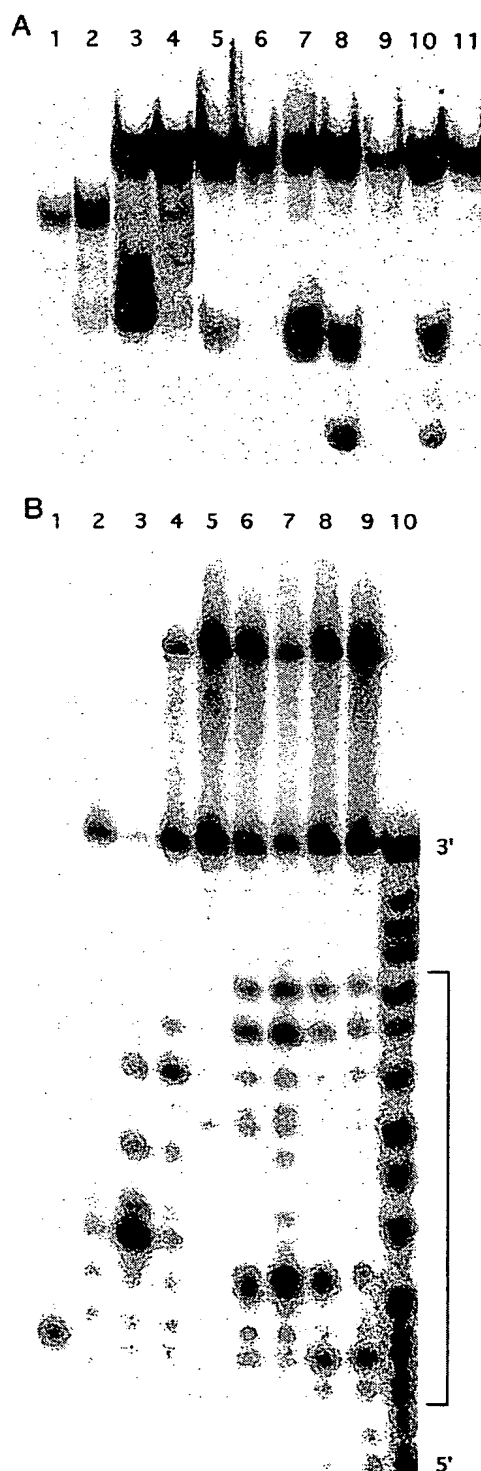


FIG. 9. Analysis of dsRNA oligonucleotide digestion products by native polyacrylamide gel electrophoresis. A, antisense and sense oligonucleotides were preannealed and incubated with the cellular extracts as described under "Materials and Methods." Polyacrylamide gel analysis of the digestion products was performed as described under "Materials and Methods." Sense strand RNA alone (lane 1) and digested with RNase V1 (lane 2) are shown. RNase V1 digestion of single strand sense oligonucleotide was performed in 10 μ l containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 10⁴ cpm RNA, and

potential potency of these RNA gap mer compounds for other sites compared with RNase H active oligonucleotides. The target site in codon 12 of Ha-Ras is one of the most RNase H-sensitive sites we have identified. A phosphorothioate oligodeoxynucleotide to that site typically displays an IC₅₀ of approximately 50 nM in T24 cells (22). The IC₅₀ for the 9-base RNA gap mer oligonucleotide was approximately 200 nM, suggesting that this activity is capable of degrading this site nearly as well as RNase H.

The selective inhibition of mutated genes such as the *ras* oncogene necessitates antisense hybridization in the coding region of the mRNA. This requires either a high affinity interaction between oligonucleotide and mRNA to prevent displacement of the oligonucleotide by the polysome or rapid degradation of the target mRNA. RNA gap mer oligonucleotides, being inherently higher in affinity than oligodeoxynucleotides and being able to take advantage of a cellular dsRNase activity, may satisfy both these criteria. Identification of sites that are differentially sensitive to RNase H and to dsRNase activities will increase the number of potential target sites on a given mRNA for antisense oligonucleotides.

It is clear that an activity capable of degrading dsRNA must be carefully regulated, since dsRNA and stem loop structures abound in all cells and uncontrolled cleavage of such substrates would surely be toxic. Mechanisms of regulation may include direct inhibitors and activators, cellular compartmentalization, and regulation by cellular signal transduction pathways. One such pathway that could potentially be involved is the dsRNA-activated protein kinase pathway (33). The kinase p68, which is induced by dsRNA or interferon, phosphorylates the eukaryotic translation initiation factor 2, which results in translational inhibition.

Further purification, characterization, and cloning of the dsRNase activity presented here will be required to increase understanding of its cellular function and regulation. Clearly, the enzyme(s) may play important roles in the intermediary metabolism of RNA and may be involved in the degradation of RNA species targeted by natural antisense transcripts. Drugs

0.5 unit of RNase V1. RNase V₁ digestion of dsRNA was prepared as above with the exception that 10⁴ cpm of sense oligonucleotide was preannealed with 10 nM antisense oligonucleotide prior to digestion with 2 \times 10⁻² units of RNase V1 (lane 3) and 2 \times 10⁻³ units of RNase V1 (lane 4). RNase reactions were incubated at 37 °C for 5 min. The digestion patterns for the dsRNA oligonucleotide incubated with the various cellular extracts are as follows: unpurified nuclear extract incubated for 240 min (lane 5); unpurified nuclear extract incubated for 240 min in the absence of MgCl₂ (lane 6); unpurified cytosolic extract incubated for 240 min (lane 7); ion-exchange purified cytosolic extract incubated for 240 min (lane 8); ion-exchange purified cytosolic extract incubated for 240 min in the absence of MgCl₂ (lane 9); ion-exchange and gel filtration-purified cytosolic extract incubated for 240 min (lane 10); ion-exchange and gel filtration-purified cytosolic extract incubated for 240 min in the absence of MgCl₂ (lane 11). B, analysis of dsRNA oligonucleotide digestion products by denaturing polyacrylamide gel electrophoresis. The bracketed region indicates the position of the RNA gap. RNase A and V1 digestions of single strand sense oligonucleotide were performed in 10 μ l containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 10⁴ cpm of ³²P-labeled RNA and 5 \times 10⁻⁴ units of RNase A (lane 1) or 2 \times 10⁻² units of RNase V₁ (lane 2). RNase V₁ digestion of dsRNA was performed as described above at 2 \times 10² units (lane 3) or 2 \times 10³ units (lane 4). The digestion patterns for the dsRNA oligonucleotide incubated with the various cellular extracts are as follows: unpurified nuclear extract incubated for 0 min (lane 5); unpurified nuclear extract incubated for 240 min (lane 6); unpurified cytosolic extract incubated for 240 min (lane 7); ion-exchange-purified cytosolic extract incubated for 240 min (lane 8); ion-exchange and gel filtration-purified cytosolic extract incubated for 240 min (lane 9). The base hydrolysis ladder was prepared by incubation of the 10⁴ cpm RNA at 90 °C for 5 min in 10 μ l containing 100 mM sodium carbonate, pH 9.0 (lane 10).

designed to take advantage of this mechanism may help increase the scope of antisense-based therapeutics.

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